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Temporal–spatial characterization of chicken clock genes: circadian expression in retina, pineal gland, and peripheral tissues

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Abstract

The molecular core of the vertebrate circadian clock is a set of clock genes, whose products interact to control circadian changes in physiology. These clock genes are expressed in all tissues known to possess an endogenous self-sustaining clock, and many are also found in peripheral tissues. In the present study, the expression patterns of two clock genes, *cBmal1* and *cMOP4,* were examined in the chicken, a useful model for analysis of the avian circadian system. In two tissues which contain endogenous clocks – the pineal gland and retina – circadian fluctuations of both *cBmal1* and *cMOP4* mRNAs were observed to be synchronous; highest levels occurred at Zeitgeber time 12. Expression of these genes is also rhythmic in several peripheral tissues; however, the phases of these rhythms differ from those in the pineal gland and retina: in the liver the peaks of *cMOP4* and *cBmal1* mRNAs are delayed 4–8 h and in the heart they are advanced by 4 h, relative to those in the pineal gland and retina. These results provide the first temporal characterization of *cBmal1* and *cMOP4* mRNAs in avian tissues: their presence in avian peripheral tissues indicates they may influence temporal features of daily rhythms in biochemical, physiological, and behavioral functions at these sites.

Keywords: *Bmal1*, circadian rhythm, *MOP4*, peripheral oscillators, pineal gland, retina.

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Most biological processes exhibit rhythmic changes in intensity with periods of about 24 h. Among these, some are known to persist in the absence of lighting or other environmental cues, reflecting the presence of endogenous clock mechanisms that drive these changes; these rhythms are referred to as 'circadian'. Such endogenous clock systems synchronize biological processes to the 24-h solar cycle and optimize daily changes in an organism's physiology to daily changes in their environment.

In mammals, most circadian rhythms reflect the action of a master clock in the brain, located in the hypothalamic suprachiasmatic nuclei (SCN; Klein *et al.* 1991). In contrast, avian circadian rhythms are regulated by a multiple oscillatory system that consists of endogenous clocks in the retina, the pineal gland, and the hypothalamus (Cassone and Menaker 1984; Gwinner and Brandstatter 2001; Underwood *et al*. 2001). These components interact with each other by humoral and neural mechanism to constitute a circadian pacemaker system.

One of the critical functions of the circadian system is to precisely control the melatonin rhythm, which is done by regulating the activity of serotonin *N*-acetyltransferase [arylalkylamine *N*-acetyltransferase (AANAT)], the penultimate enzyme in melatonin synthesis (Klein *et al*. 1997). Melatonin plays an important role in controlling rhythmic physiology in

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Abbreviations used: AANAT, arylalkylamine *N*-acetyltransferase; bHLH-PAS, basic helix-loop-helix PER-ARNT-SIM; *Bmal1*, brain and muscle ARNT-like protein 1; LD, light–dark; *MOP4*, member of the PAS super family protein 4; NPAS2, neuronal PAS domain protein 2; RT–PCR, reverse transcription–polymerase chain reaction; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

birds and is considered an important output pathway of the circadian system in these animals (Zimmerman and Menaker 1979). The primary site of the synthesis of circulating melatonin is the pineal gland. In birds, the circadian patterns of melatonin synthesis and AANAT activity are regulated by endogenous oscillators, one located in the hypothalamus at a site that is functionally equivalent to the mammalian SCN. It acts on the pineal gland via a neural pathway to activate adrenergic mechanisms, which inhibit melatonin synthesis during the day (Takahashi *et al*. 1989; Cassone and Lu 1994; Underwood *et al*. 2001). The other oscillator that regulates melatonin synthesis is located within pinealocytes themselves (Binkley *et al*. 1978; Deguchi 1979; Takahashi *et al*. 1980). The autonomous nature of the pinealocyte clock is evident from studies demonstrating a circadian rhythm of pineal melatonin synthesis *in vitro* (Zatz 1996; Nakahara *et al*. 1997).

A secondary site of melatonin synthesis is the retina, where a similar clock-dependent pathway regulates the melatonin rhythm (Gern and Ralph 1979; Hamm and Menaker 1980; Bernard *et al*. 1997; Iuvone *et al*. 2002). *In vivo* and *in vitro* studies have shown that an endogenous clock regulates mRNA rhythms of melatonin synthesis enzymes in both the pineal gland and retina (Green *et al*. 1996; Grève *et al*. 1996; Bernard *et al*. 1997; Klein *et al*. 1997; Chong *et al*. 1998; Bernard *et al*. 1999; Iuvone *et al*. 2002).

We are broadly interested in the regulation of melatonin production and, in the study presented here, attention was focused on molecular elements of the endogenous clock in the chicken. Endogenous clocks are now known to be highly conserved in organisms as diverse as cyanobacteria, plants, fruit flies, and mammals (Dunlap 1999; Panda *et al*. 2002a). The model that has emerged is one of interlocking transcriptional/translational feedback loops with positive and negative 'clock genes' (Hastings 2000; Harmer *et al*. 2001; Reppert and Weaver 2001). Although this model has been generally conserved, some variation in the nature of the individual molecular components of circadian clocks has been noted, indicating that an accurate understanding of clock function in any one organism may not necessarily be inferred from studies in another.

An example of such differences comes from analysis of the chicken clock. One of the genes that is part of many circadian clocks is *Bmal1,* which encodes a member of the basic helix-loop-helix/PER-ARNT-SIM (bHLS/PAS) family of transcription factors. BMAL1 acts as a positive regulator in the intracellular transcriptional/translational feedback loop at the core of the circadian clockwork in mammals (Chang and Reppert 2001). BMAL1 heterodimerizes with another clock gene product, CLOCK, to activate transcription of *Per* genes through E-box enhancer elements in their promoters (Bunger *et al*. 2000; Shearman *et al*. 2000). Whereas this body of evidence points to a BMAL1/CLOCK dimer as being important, other studies indicate that the role of CLOCK can be played by another bHLH-PAS transcription factor – MOP4 (NPAS2). Although *MOP4* mRNA is undetectable in the mammalian SCN (Shearman *et al*. 1999), in other tissues it appears to play a role in activating E-box driven transcription via heterodimerization with BMAL1 (Hogenesch *et al*. 1998; Kume *et al*. 1999; Chong *et al*. 2000). Of special significance to the current report is the finding that E-box-dependent expression of the chicken AANAT gene can be controlled by either cCLOCK/cBMAL1 or cMOP4/cBMAL1 dimers (Chong *et al*. 2000). Accordingly, it appears that CLOCK and MOP4 are BMAL1 partners and mediate the same circadian clock function in different tissues. Recent studies have shown variants of the *Bmal1* gene in human and mouse tissues (Ikeda and Nomura 1997; Yu *et al*. 1999). In addition, Okano *et al*. (2001) have identified *cBmal1b′* in chicken pineal gland. The functional importance of *Bmal1* variants is unclear.

Little is known about the physiological temporal expression of *cBmal1* and *cMOP4* mRNAs in the chicken clock and, as described in this report, we have now studied these transcripts in the chicken pineal gland and retina. In parallel, we have also studied the heart and liver, in an effort to unveil the possible existence of peripheral circadian oscillators in chickens, as has been found in other species (Oishi *et al*. 1998, 2000; Sakamoto *et al*. 1998; Whitmore *et al*. 1998, 2000; Zylka *et al*. 1998; Cermakian *et al*. 2000; Sakamoto and Ishida 2000; Glossop and Hardin 2002). Our results provide new insight into the potential function of these genes in the chicken circadian system.

Materials and methods

Animals and tissue collection

One-day-old-male chicks (White leghorn; *Gallus domesticus*, Hy-Line International, Covington, GA, USA) were housed for 2 weeks in heated brooders on a 12-h light : 12-h dark cycle [LD 12 : 12; lights on from Zeitgeber time (ZT) 0 to 12] with lights provided by cool white fluorescent tubes. Light intensity within the brooders was approximately 0.1–10 $\mu W/cm^2$. Following this, the animals were subjected to constant (24 h/day) dark (DD) or light (LL). Chicks (3–4) were killed by decapitation in LD, LL or DD at the times described in the figure legends. To examine the re-entrainment of circadian rhythms, subjects were housed for 10 days under the control LD cycle and were then exposed to a reversed LD cycle for 3 days. Subjects were then transferred to DD, and were killed at the indicated times on the second day of DD. Tissues were dissected and immediately frozen on dry ice. Dissection in 'darkness' was performed under dim red light (Wratten no. 92; < 2 min from exposure to freezing of tissue). The experimental protocols meet the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

RT–PCR analysis of *cBmal1* splice-variants

Total RNA was extracted from various chicken tissues using TriZol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using

Expand reverse transcriptase according to the manufacturers' instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA) with an oligo-dT anchoring primer, 5′-T30(A/C/g/T)(A/C/g)-3′. PCR was performed for 35 cycles using four primers that were specific for the 21-bp insert and 28-bp deletion sequence according to the chicken and mouse data (Yu *et al*. 1999).

A partial cDNA fragment for *cBmal1* was isolated from pineal cDNA using degenerate PCR and used to screen a chicken pineal cDNA library (Chong *et al*. 2000). The cloned cDNA contains a continuous open reading frame of 1902 bp encoding a 634-amino acid protein. The amino acid sequence of *cBmal1* is 93% identical to mouse *cBmal1b′* (Yu *et al*. 1999), with identical sequences in the bHLH, PAS-A and PAS-B regions. *cBmal1* corresponds to *mBmal1b′* except for an insertion of a CAG trinucleotide at nucleotide 841. Both mouse and chicken *Bmal1b′* contain an insertion of 21 bp at nucleotide 141; therefore, the cloned *cBmal1* is an ortholog of *mBmal1b′*.

Primers used were: CM3b'F: 5′-ACTGATTATCAACTTGATggCT-3′; CM3bg'F: 5′-ACTGATTATCAAGAAGGTATGG-3′; CM3bb'R: 5′-TCCCTCTGTCACAGCCCA-3′; CM3g'R: 5′-TCTGTCACAGCCCTGAGTAT-3′. The PCR products were then electrophoresed and Southern blotted. Blots were probed using the partial *cBmal1b′* cDNA (384 bp) which overlaps the amplified regions. Hybridization was carried out at 55°C, with the final wash at 50°C in 0.2 × saline–sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 15 min.

Northern blot analysis

Northern blot analysis was performed as previously described (Chong *et al*. 1998). All RNA samples were isolated from tissues dissected from individual animals except for the pineal, where three glands were pooled. Unless noted otherwise, 10 μg of total RNA were loaded per lane on a 1.2% agarose formaldehyde (0.7 M) gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell, Dassel, Germany). Blots were probed with random-primed ^{32}P-labeled cDNA of either *cBmal1* (1.9 kb) or *cMOP4* (1.2 kb). Data were normalized for variations in RNA loading and transfer efficiency by subsequently probing the northern blots with a 2-kb human β-actin cDNA (Clontech, Palo Alto, CA, USA). All probes were hybridized to northern blots at 68°C in QuikHyb (Stratagene, La Jolla, CA, USA), and the final wash was done at 60°C in 0.1 × SSC/0.1% SDS for 15 min Hybridized blots were exposed on X-ray film and their images scanned and analyzed using the Image βPC1 software (Scion Image, NIH, USA). Transcript sizes were estimated by comparison with standard RNA markers (RNA Molecular Weight Marker I; Roche Molecular Biochemicals).

Real-time RT–PCR

Total RNA was extracted by a silica filter-binding method using the RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription (RT) was performed on total RNA (2 μg) preparations using M-MLV reverse transcriptase (Invitrogen) and oligo-dT as the primer (Invitrogen). cDNA fragments of *cBmal1* (approximately 1.5 kb), *cMOP4* (approximately 1.0 kb) and *cGAPDH* (approximately 0.9 kKb) were generated by PCR, gel purified, and subcloned into the pPCR-Script Amp SK(+) cloning vector (Stratagene). These cDNAs were used as standards in the real-time RT–PCR assays.

Two microliters of cDNA from each sample were amplified using the following primers: *cBmal1*(GenBank accession no. AF205219), forward 5′-ATGGAAGACTGGACTACGCAGAC-3′ and reverse 5′-ATGCTGGACTGCCATTCTCAATAC-3′; *cMOP4*: (GenBank accession no. AF 396828), forward 5′-CAAGCCAGAGTTCATCGTGTGCA-3′ and reverse 5′-TGAGCCTGTGTGCAGATAAGTCC-3′; and *cGAPDH* (GenBank accession no. K01458); forward 5′-AGTCCAAGTGGTGGCCATCAATG-3′, reverse 5′-TTATCACCACCCTTCAGATGAGC-3′.

For the amplification, reactions were performed in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) with a 25-μL total volume containing cDNA, 1× SYBR Green PCR Master mix, and 300 nM gene-specific forward and reverse primers. Samples were incubated at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and extension at 95°C, 53°C, and 72°C for 30 s each, respectively. PCR products were checked by agarose electrophoresis. The primers yielded products of 191 bp, 349 bp, and 270 bp for *cBmal1*, *cMOP4*, and *cGAPDH*, respectively. The quantification of transcript level was performed by comparing the threshold cycle for amplification of the unknown to those of six concentrations of standard cDNAs for *cBmal1*, *cMOP4*, and *cGAPDH*. Each sample was assayed in triplicate and normalized to the expression of the housekeeping gene, *cGAPDH*.

Statistical analysis

Statistical analysis among groups was performed using one-way analysis of variance (ANOVA) with Student–Newman–Keuls multiple comparison test where applicable.

Results

Twenty-four-hour rhythms in retinal and pineal *cBmal1* and *cMOP4* transcripts

The retina and pineal gland were found to express robust rhythms of *cBmal1* and *cMOP4* mRNA, which persist for two circadian cycles in constant illumination. Peak levels of both transcripts in retina and pineal gland occur at approximately ZT 12 in LD (Figs 1 and 2). Amplitudes of the *cBmal1* mRNA are 10- and 40-fold in the retina and pineal gland, respectively, whereas the *cMOP4* mRNA rhythm amplitudes are six- and 14-fold, respectively, in the two tissues.

The issue of whether the daily rhythm in *cBmal1* and *cMOP4* transcripts is dependent on L to D or D to L transitions was examined by maintaining animals in either constant light (LL) or constant darkness (DD) for 48 h (Figs 1 and 2). Under these conditions, the daily rhythms in both transcripts in the retina and pineal gland were found to persist, with little change in either amplitude or phase, other than a lengthening of the duration of elevated levels of retinal *cBmal1* during the second subjective night in LL (Fig. 1b). Northern blot analyses further demonstrated that *cBmal1* and *cMOP4* mRNAs are rhythmically expressed in the retina in LL and DD, with high levels in the early subjective night

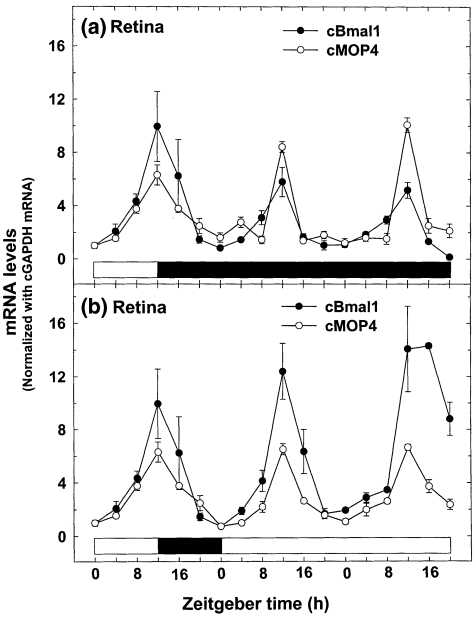

Fig. 1 Temporal expression of *cBmal1* (●) and *cMOP4* (○) mRNAs in the chicken retina. The circadian changes illustrated are representative of two different experiments performed under constant darkness (DD, a) and constant light (LL, b). One-day-old chicks were housed in a 12-h light–dark (LD) cycle (lights on at ZT 0) for 2 weeks and later kept under DD or LL for 2 days. Tissues were collected in LD, LL and DD at ZT 0, 4, 8, 12, 16, and 20. Relative mRNA levels in the tissues were quantified by real-time RT–PCR. Each data point represents *cBmal1* or *cMOP4* mRNA, normalized to *cGAPDH* mRNA, of four retinas from four individual chickens and expressed relative to the ZT 0-values in LD. □, Times of lights-on; ■, times of darkness. Analysis of variance (ANOVA) indicated a significant effect of time-of-day ($p < 0.001$) on *cBmal1* and *cMOP4* mRNA levels for each day under LD, DD, and LL. Post-hoc analyses of daily fluctuations of mRNA levels indicated the following significant differences between ZT 0 and other times of day, indicative of daily rhythmicity. In LD: *cBmal1* – ZT 12 $p < 0.001$, ZT 16 $p = 0.046$; *cMOP4* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$. On day 1 of DD: *cBmal1* – ZT 8 $p = 0.049$, ZT 12 $p < 0.001$; *cMOP4* – ZT 12 $p < 0.001$. On day 2 of DD: *cBmal1* – ZT 8 $p = 0.002$, ZT 12 $p < 0.001$; *cMOP4* – ZT 12 $p < 0.001$. On day 1 of LL: *cBmal1* – ZT 12 $p < 0.001$, ZT 16 $p = 0.023$; *cMOP4* – ZT 8 $p = 0.005$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$. On day 2 of LL: *cBmal1* – ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p = 0.025$; *cMOP4* – ZT 8 $p = 0.036$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$.

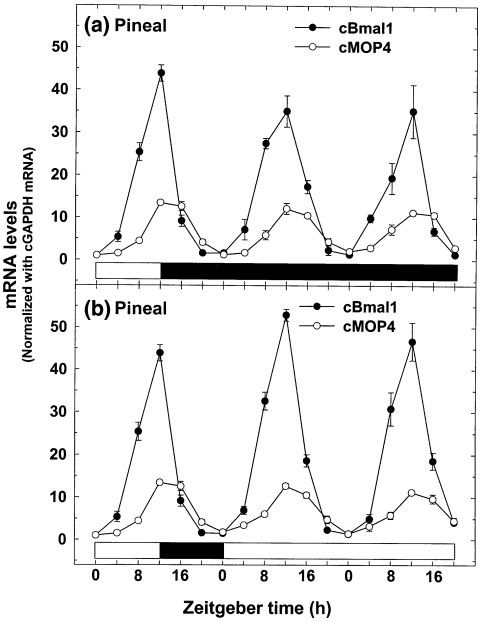

Fig. 2 Temporal expression of *cBmal1* (●) and *cMOP4* (○) mRNAs in the chicken pineal. The circadian changes illustrated are representative of two different experiments performed under constant darkness (DD, a) and constant light (LL, b). One-day-old chicks were housed in a 12-h light : 12-h dark (LD) cycle (lights on at ZT 0) for 2 weeks and later kept under DD or LL for 2 days. Tissues were collected in LD, LL and DD at ZT 0, 4, 8, 12, 16, and 20. Relative mRNA levels in the tissues were quantified by real-time RT–PCR. Each data point represents *cBmal1 or cMOP4* mRNA, normalized to *cGAPDH* mRNA, of four pineal glands, expressed relative to the ZT 0-values in LD. □, Times of lights-on; ■, darkness. ANOVA indicated a significant effect of time-of-day ($p < 0.001$) in *cBmal1* and *cMOP4* mRNA levels on each day under LD, DD, and LL. Post-hoc analyses of daily fluctuations of mRNA levels indicated the following significant differences between ZT 0 and other times of day, indicative of daily rhythmicity. In LD: *cBmal1* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p = 0.001$; *cMOP4* – ZT 8 $p = 0.001$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p = 0.002$. On day 1 of DD: *cBmal1* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 p,0.001; *cMOP4* – ZT 8 $p = 0.003$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p = 0.034$. On day 2 of DD: *cBmal1* – ZT 8 $p = 0.009$, ZT 12 $p < 0.001$; *cMOP4* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$. On day 1 of LL: *cBmal1* – ZT 4 $p = 0.017$, ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$; *cMOP4* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p = 0.005$. On day 2 of LL: *cBmal1* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p = 0.001$; *cMOP4* – ZT 8 $p = 0.005$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p = 0.036$.

(Fig. 3). Two transcripts were observed for each gene: 2.6 kb and 4.6 kb for *cBmal1* and 4.7 kb and 7 kb for *cMOP4*. The levels of the small and large transcripts of both genes varied on a 24-h basis.

Photic entrainment of retinal *cBmal1* mRNA rhythm

The ability of light to re-entrain the rhythm of *cBmal1* mRNA was examined by housing chicks for 3 days in a reversed LD cycle and then 'releasing them' into DD. On the second day of DD, robust rhythms of *cBmal1* mRNA were observed in retinas exposed to either the normal or reversed LD cycle. However, the phase of this *cBmal1* mRNA rhythm following light cycle reversal was 180° out of phase to that of the animals maintained on the original LD cycle, indicative of a complete re-entrainment of the circadian oscillation (Fig. 4). A similar pattern of re-entrainment has been reported for the TPH mRNA rhythm in chicken pineal and retina (Chong *et al.* 1998).

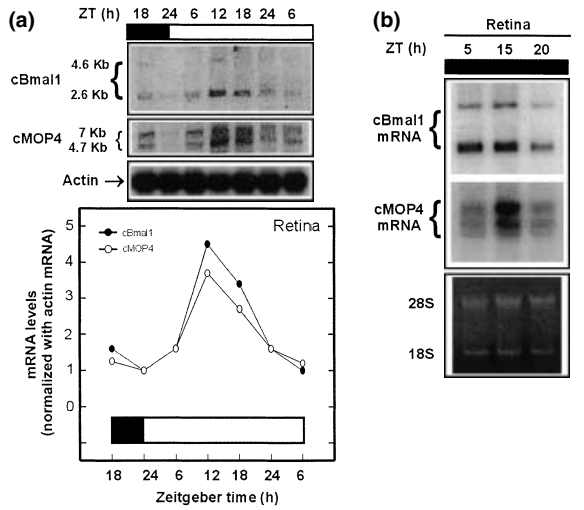

Fig. 3 Northern blot analyses of *cBmal1* and *cMOP4* transcripts in the retina in (a) constant light (LL) and (b) constant darkness (DD). (a, top) Representative northern blots showing expression of *cBmal1* and *cMOP4* transcripts in the chicken retina studied in LL. Numbers above each lane correspond to the ZT points at which RNA samples were prepared. (a, bottom) Semi-quantitative analysis of rhythmic *cBmal1* and *cMOP4* expression; the abundance of *cBmal1* (2.6 kb) and *cMOP4* (4.7 kb) transcripts has been normalized to actin mRNA. *cBmal1* and *cMOP4* mRNA levels clearly exhibit rhythmic variations in abundance with high levels at ZT 12. All experiments were repeated with similar results on independently obtained samples. (b) Representative northern blots showing expression of *cBmal1* and *cMOP4* transcripts in the chicken retina studied in DD. Numbers above each lane correspond to the ZT points at which RNA samples were prepared. Equivalent amounts of RNA were used in each case, as assessed by ethidium bromide staining of 28S and 18S ribosomal RNA. Similar results were obtained in two different experiments on independently obtained samples.

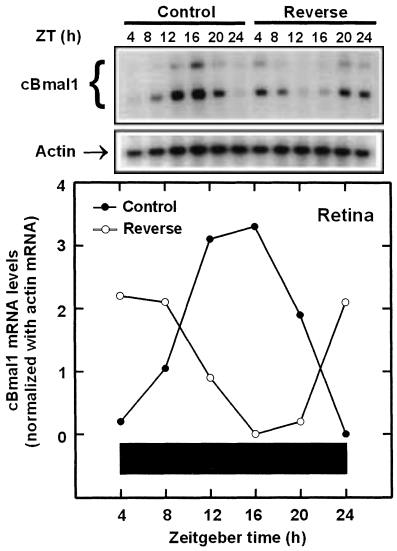

Fig. 4 Re-entrainment of the circadian rhythm of *cBmal1* mRNA levels. All animals were initially entrained to the same LD cycle. One set of chicks was subsequently kept for three full cycles on a reversed 12-h light : 12-h dark cycle and transferred to DD. *cBmal1* mRNA levels were determined on the second day of DD. Control animals (●) maintained on the original LD cycle were placed in DD and *cBmal1* mRNA levels were determined in parallel with the animals of the reversed light cycle (○). Retinas were collected at the indicated times and processed for *cBmal1* and β-actin RNAs. Zeitgeber time in this graph refers to the original entrainment LD cycle. Each data point represents the level of *cBmal1* mRNA, corrected for actin mRNA, of three retinas. Similar results were obtained in a separate experiment.

Circadian nature of peripheral rhythms

To test whether the daily abundance of *cBmal1* and *cMOP4* transcripts changes in tissues besides the pineal gland and retina, we performed northern blots using samples collected at three time points during a DD cycle (Fig. 5). Expression of *cBmal1* mRNA was observed in hypothalamus and peripheral tissues such as the liver, skeletal muscle, and intestine (Fig. 5a), with high levels at ZT 15 compared to ZT 5 or ZT 20. Relative to the pineal gland, expression levels were low in all peripheral tissues examined, except the intestine. Low levels of chicken *cMOP4* mRNA are also rhythmically expressed in the hypothalamus and liver (Fig. 5b), with high levels at ZT15.

Rhythmic expression of *cBmal1* and *cMOP4* mRNAs was observed in the liver and heart (Fig. 6), although with different temporal profiles of expression. The liver *cBmal1* transcript rhythm had an amplitude of approximately 18-fold and a peak at ZT 16 in LD (Fig. 6a). The amplitude increased to approximately 28-fold in the subsequent DD cycle (Fig. 6a). The liver *cMOP4* mRNA rhythm had a broad peak between ZT 12 and ZT 20 and an amplitude of approximately 14-fold in LD (Fig. 6a). In DD, the amplitude increased to approximately 32-fold, with a peak at ZT 16 (Fig. 6a). Highest levels of *cBmal1* and *cMOP4* transcripts were detected at an earlier time point (ZT 8) in heart compared to the liver, pineal, and retinal profiles (Fig. 6b). The amplitudes of the heart *cBmal1* mRNA rhythms were sixfold in LD and ninefold in DD; the amplitudes of the *cMOP4* transcript rhythms were sevenfold and 15-fold in LD and DD, respectively. These observations provide direct evidence for the existence of peripheral oscillators in chicken.

Localization of splice variants of *cBmal1* in pacemaker and peripheral tissues

The cDNA of possible splice variants of *cBmal1* were characterized by the presence or absence of a 21-bp insert and a 28-bp deletion relative to *mBmal1b* (Yu *et al.* 1999). The tissue distributions of the splice variants of *cBmal1* were examined by RT–PCR using specific primers directed against those sequences. We designed two upstream primers, CM3b'F and CM3bg'F, specific to the cDNA regions with

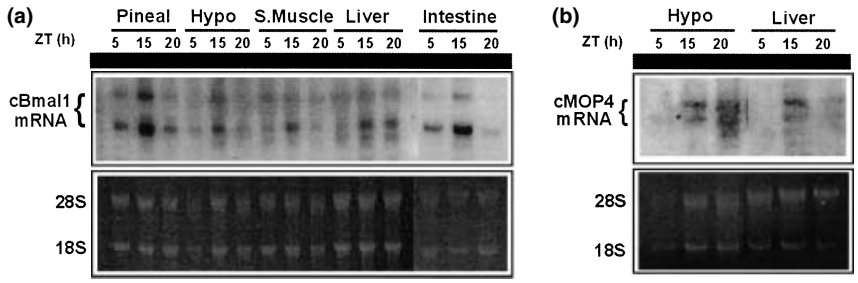

Fig. 5 Temporal expression of clock genes in chicken tissues in constant darkness. (a) *cBmal1*. Representative northern blot showing expression in pineal, hypothalamus (hypo), and peripheral tissues at three different time points. (b) *cMOP4*. Representative northern blot showing expression in hypothalamus and liver at three different time points. Numbers above each lane correspond to the ZT points at which RNA samples were prepared. Equivalent amounts of RNA were used in each case, as assessed by ethidium bromide staining of 28S and 18S ribosomal RNAs. Similar results were obtained in two different experiments on independently obtained samples.

and without the 21-bp insert, and two downstream primers, CM3bb'R and CM3g'R, specific to the cDNA regions with and without the 28-bp deletion, respectively (Fig. 7a). An aliquot of all PCR products were electrophoresed, transferred onto nylon membrane, and then probed with a ^{32}P-labeled *cBmal1* (384 bp) cDNA spanning the electrophoresed products. All splice variants were widely expressed but their abundance seems to be quite different in various tissues (Fig. 7B). Southern blotting of PCR showed that *cBmal1b′* appears to be the most abundant splice variant, with low levels of *cBmal1b* and *cBmal1g′* (Fig. 7b).

Discussion

In the present study, we describe the expression of the circadian clock genes, *cBmal1* and *cMOP4*, which showed robust daily oscillations that persist in constant darkness and/or constant light. Previously, we demonstrated that *cClock*, *cBmal1*, and *cMOP4*, are co-ordinately expressed in the chicken retina and pineal gland (Chong *et al.* 2000). As indicated in the Introduction, the circadian rhythm of melatonin biosynthesis in chicken retina and pineal gland is driven in part by clock-controlled gene expression of AANAT (Bernard *et al*. 1997), a key regulatory enzyme in the melatonin biosynthetic pathway (Klein *et al.* 1997); clock gene heterodimers (cBMAL1/cCLOCK and cBMAL1/cMOP4) activate the AANAT-E-box and enhance transcription (Chong *et al.* 2000). The phase of the rhythms of *cBmal1* and *cMOP4* mRNAs is consistent with such a role in regulation of AANAT expression. The rhythmic increase in the expression of these clock genes precede that of AANAT mRNA by approximately 4 h in retina and pineal gland (Bernard *et al*. 1997; Chong *et al*. 2000; Haque *et al*. 2002). Also, the relative amplitudes of the rhythms are consistent with this proposed regulatory role of the clock genes in AANAT expression. The amplitudes of the *cBmal1*, *cMOP4*, and AANAT transcript rhythms are all higher in pineal gland than in retina (Bernard *et al*. 1997; the present results). BMAL1 is generally considered to be an essential component of the core oscillatory mechanism of circadian clocks in mammals and birds (Chang and Reppert 2001; Fukada and Okano 2002). Thus, the rhythmic expression of *Bmal1* provides a direct link from the circadian clock to the output pathway.

We demonstrate in the present study that the positive clock elements, *cBmal1* and *cMOP4*, are rhythmically expressed in multiple peripheral organs of chickens, suggesting a widespread presence of multiple clocks in this species as appears to be the case in other vertebrates (Oishi *et al*. 1998, 2000; Sakamoto *et al*. 1998; Whitmore *et al*. 1998, 2000; Zylka *et al*. 1998; Cermakian *et al*. 2000; Sakamoto and Ishida 2000; Glossop and Hardin 2002). Multiple clocks in chickens may be working independently, as observed in the zebrafish (Whitmore *et al*. 2000), and/or governed by the central pacemaker system, as reported for mammals (Reppert and Weaver 2002). At present, the underlying mechanism of regulation for these clock genes in different chicken tissues is unclear. Relative to the *cBmal1* and *cMOP4* transcript rhythms in retina and pineal gland, the rhythms in the heart were phase advanced by approximately 4 h, while those in the liver were phase delayed by 4–8 h. Differences in phase of clock gene transcript rhythms in SCN and peripheral tissues have been observed in mammals (Zylka *et al.* 1998; Balsalobre *et al.* 2000; Storch *et al*. 2002). It is conceivable that different output pathways and organ-specific genes are being activated at different times of the day. In support of this, circadian microarray analyses using mouse SCN, liver, and heart samples have demonstrated little overlap of rhythmically expressed transcripts among these different tissues (Panda *et al*. 2002b; Storch *et al*. 2002). Young *et al*. (2001) observed a robust rhythm of *Bmal1* in rat heart with high levels at ZT 0, which is antiphase to the circadian profile in chicken heart reported in the present study. This may reflect a time-of-day difference between activity level of the nocturnal rodent and the diurnal chicken.

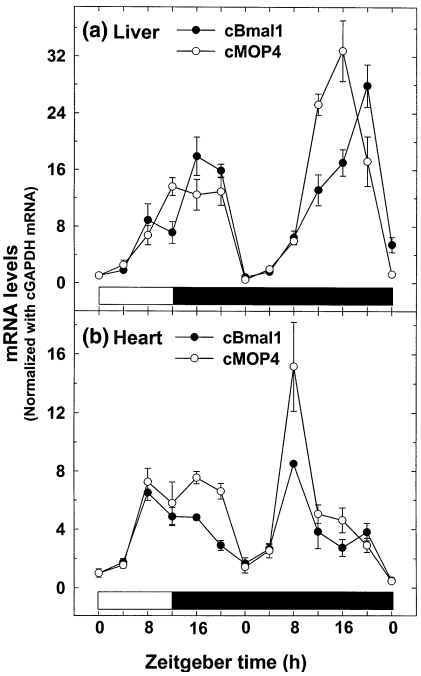

Fig. 6 Temporal expression of *cBmal1* (●) and *cMOP4* (○) mRNAs in the chicken liver (a) and heart (b) under LD and DD. One-day-old chicks were housed in a 12-h light : 12-h dark (LD) cycle (lights on at ZT 0) for 2 weeks and later kept under DD for 2 days. Tissues were collected in LD and DD at ZT 0, 4, 8, 12, 16, and 20. Relative mRNA levels in the tissues were quantified by real-time RT–PCR. Each data point represents *cBmal1* or *cMOP4* mRNA, corrected for *cGAPDH* mRNA, of tissues from four individual chickens, expressed relative to the ZT 0 (LD) values. □, Lights-on; ■, darkness. ANOVA indicated a significant effect of time-of-day ($p < 0.001$) in *cBmal1* and *cMOP4* mRNA levels under LD and DD in heart and liver. Post-hoc analyses of daily fluctuations of mRNA levels indicated the following significant differences between ZT 0 and other times of day, indicative of daily rhythmicity. In heart under LD: *cBmal1* – ZT 8 $p < 0.001$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p = 0.01$; *cMOP4* – ZT 8 $p < 0.001$, ZT 12 $p = 0.001$, ZT 16 $p < 0.001$, ZT 20 $p < 0.001$. In heart under DD: *cBmal1* – ZT 8 $p < 0.001$; *cMOP4* – ZT 8 $p < 0.001$. In liver under LD: *cBmal1* – ZT 8 $p = 0.016$, ZT 12 $p = 0.042$, ZT 16 $p < 0.001$, ZT 20 $p < 0.001$; *cMOP4* – ZT 8 $p < 0.03$, ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p < 0.001$. In liver under DD: *cBmal1* – ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p < 0.001$; *cMOP4* – ZT 12 $p < 0.001$, ZT 16 $p < 0.001$, ZT 20 $p < 0.001$.

The present study revealed splice-variants of *cBmal1* distributed throughout the body; the most abundant is *cBmal1b′*. It is interesting to observe that the splice sites in the mammalian *Bmal1* gene and the chicken orthologs are identical, indicating conservation during phylogenesis. At present, the functional significance of these splice variants is unclear. Nucleotide deletions have produced a frame-shift in the splice variant *cBmal1g′*, which leads to a premature stop codon after the PASA domain (Fig. 7). The putative gene product of *cBmal1g′* will therefore be truncated and it is conceivable that this may act as an endogenous dominant-negative for the functional *cBmal1b′*. In support of this, Takahata *et al*. (2000) have demonstrated that the mouse *Bmal1* contains a transcriptional activation domain at the most C-terminal end (amino acids 582–626). Deletion of this small region of mouse *BMAL1* abolished the transactivation activity (with CLOCK) using the *mPer1* E-box-luciferase reporter assay, but had no effect on DNA-binding capacity.

The present study also demonstrates for the first time the expression profiles of *cMOP4* mRNA in a non-mammalian species. MOP4, which shares high homology at the amino acid level with CLOCK (Hogenesch *et al*. 1997; Zhou *et al*. 1997), heterodimerizes with BMAL1 and promotes E-box activation of clock genes like *Per1* and clock-controlled genes like AANAT and vasopressin (Hogenesch *et al*. 1998; Kume *et al*. 1999; Chong *et al*. 2000; McNamara *et al*. 2001). However, the lack of *MOP4* expression in SCN of neonatal and adult mice has limited consideration of its involvement in the mammalian circadian feedback loop (Shearman *et al*. 1999). In the present study, rhythmic co-expression of *cBmal1* and *cMOP4* mRNAs was observed in all chicken tissues examined, including central pacemaker tissues (hypothalamus, pineal gland, retina). The co-ordinated peaks of *cBmal1* and *cMOP4* expression may have special utility in circadian gene activation in the chicken. In contrast to the rhythmic expression of *cMOP4* mRNA, *cClock* expression displays low amplitude or no rhythms in the chicken (Larkin *et al*. 1999; Chong *et al*. 2000; Okano *et al*. 2001). Thus, co-ordinate rhythms of *cBmal1* and *cMOP4* may play a more important role in circadian clock function or output than cCLOCK/cBMAL1. Recent data in mammals also indicate that functional CLOCK is not required for the entrainment of peripheral clocks to restricted feeding (Oishi *et al*. 2002). It is conceivable that novel target genes of cMOP4/cBMAL1 heterodimers exist in the chicken and these might be tissue- and/or time-specific outputs. It is worth noting that the peak levels of *cMOP4* mRNA in DD is significantly higher than those in LD in liver and heart (Fig. 6), but not in the pineal gland and retina (Figs 1 and 2). At present, the explanation and significance of this is unknown.

The core molecular pacemaker is well defined in the rodent, although the molecular outputs that ultimately regulate circadian control of cellular physiology, organ biology, and systems-level behaviour are still poorly understood. DNA microarray analysis (Akhtar *et al*. 2002; Panda *et al*. 2002b; Storch *et al*. 2002) indicates that *MOP4* mRNA expression in the mouse liver is low; moreover, it is apparently arrhythmic. A large-scale analysis of the mouse transcriptomes using microarrays has also shown that *MOP4* mRNA is poorly expressed in the mouse (Su *et al*. 2002), further demonstrating the unique properties of the chicken as a circadian model system for the examination of the putative clock gene, *MOP4*.

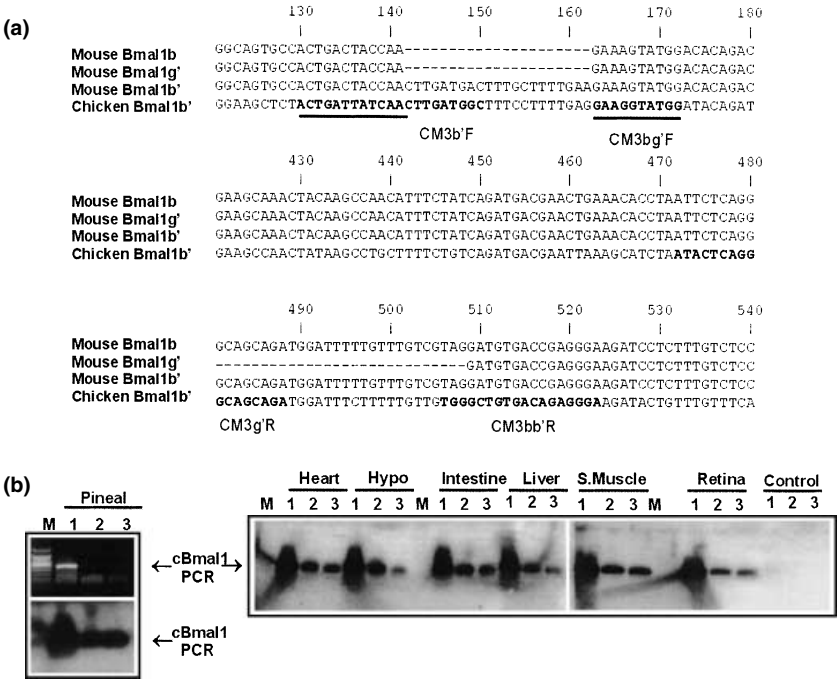

Fig. 7 Tissue distribution of *cBmal1* splice variants. (a) Schematic representation of the positions of the primers used (in bold) in RT–PCR. The primer CM3bg'F, which spans the flanking sequence of the 21 bp insert, is underlined. (b) RT–PCR Southern blot analysis of *cBmal1* variant mRNA expression in chicken tissues. Lane 1, *cBmal1b'* (393 bp); lane 2, *cBmal1b* (372 bp); lane 3, *cBmal1g'* (344 bp); and lane M, 100-bp ladder. Control had no cDNA template.

In conclusion, the widespread expression of circadian rhythms and clock genes across organ systems underlines the relevance of temporal integration to normal physiology. Further examination of the chicken circadian system provides not only a novel model in respect of the *MOP4* gene but also an interesting perspective for comparative analysis at the level of the whole animal. Appreciation of the molecular basis of circadian timing and its integration at the level of the whole organism will therefore have widespread applicability to understanding normal physiology and disease.

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